

**ATTACHMENT A**  
**FRESHWATER ACUTE**  
**TOXICITY TEST PROCEDURE AND PROTOCOL**

**I. GENERAL REQUIREMENTS**

The permittee shall conduct acceptable acute toxicity tests in accordance with the appropriate test protocols described below:

- **Daphnid (Ceriodaphnia dubia) definitive 48 hour test.**
- **Fathead Minnow (Pimephales promelas) definitive 48 hour test.**

Acute toxicity test data shall be reported as outlined in Section VIII.

**II. METHODS**

Methods should follow those recommended by EPA in:

Weber, C.I. et al. Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, Fourth Edition. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH. August 1993, EPA/600/4-90/027F.

Any exceptions are stated herein.

**III. SAMPLE COLLECTION**

A discharge sample shall be collected. Aliquots shall be split from the sample, containerized and preserved (as per 40 CFR Part 136) for chemical and physical analyses required. The remaining sample shall be measured for total residual chlorine and dechlorinated (if detected) in the laboratory using sodium thiosulfate for subsequent toxicity testing. (Note that EPA approved test methods require that samples collected for metals analyses be preserved immediately after collection.) Grab samples must be used for pH, temperature, and total residual chlorine (as per 40 CFR Part 122.21).

Standard Methods for the Examination of Water and Wastewater describes dechlorination of samples (APHA, 1992). Dechlorination can be achieved using a ratio of 6.7 mg/L anhydrous sodium thiosulfate to reduce 1.0 mg/L chlorine. A thiosulfate control (maximum amount of thiosulfate in lab control or receiving water) should also be run.

All samples held overnight shall be refrigerated at 4°C.

#### IV. DILUTION WATER

A grab sample of dilution water used for acute toxicity testing shall be collected from the receiving water at a point upstream of the discharge free from toxicity or other sources of contamination. Avoid collecting near areas of obvious road or agricultural runoff, storm sewers or other point source discharges. An additional control (0% effluent) of a standard laboratory water of known quality shall also be tested.

If the receiving water diluent is found to be, or suspected to be toxic or unreliable, an alternate standard dilution water of known quality with a hardness, pH, conductivity, alkalinity, organic carbon, and total suspended solids similar to that of the receiving water may be substituted **AFTER RECEIVING WRITTEN APPROVAL FROM THE PERMIT ISSUING AGENCY(S)**. Written requests for use of an alternate dilution water should be mailed with supporting documentation to the following address:

Director  
Office of Ecosystem Protection  
U.S. Environmental Protection Agency, Region 1  
One Congress Street  
Suite 1100 (CAA)  
Boston, MA 02114-2023

It may prove beneficial to have the proposed dilution water source screened for suitability prior to toxicity testing. EPA strongly urges that screening be done prior to set up of a full definitive toxicity test any time there is question about the dilution water's ability to support acceptable performance as outlined in the 'test acceptability' section of the protocol.

#### V. TEST CONDITIONS

The following tables summarize the accepted daphnid and fathead minnow toxicity test conditions and test acceptability criteria:

##### **EPA NEW ENGLAND RECOMMENDED EFFLUENT TOXICITY TEST CONDITIONS FOR THE DAPHNID, CERIODAPHNIA DUBIA 48 HOUR ACUTE TESTS<sup>1</sup>**

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|---------------------|---------------------------------|
| 1. Test type        | Static, non-renewal             |
| 2. Temperature (°C) | 20 ± 1° C or 25 ± 1° C          |
| 3. Light quality    | Ambient laboratory illumination |

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|----------------------|----------------------------|
| 4. Photoperiod       | 16 hour light, 8 hour dark |
| 5. Test chamber size | Minimum 30 ml              |

6. Test solution volume	Minimum 25 ml
7. Age of test organisms	1-24 hours (neonates)
8. No. daphnids per test chamber	5
9. No. of replicate test chambers per treatment	4
10. Total no. daphnids per test concentration	20
11. Feeding regime	Feed YCT and <u>Selenastrum</u> while holding organisms prior to initiating test as per manual.
12. Aeration	None
13. Dilution water <sup>2</sup>	Receiving water, other surface water, synthetic soft water adjusted to the hardness and alkalinity of the receiving water (prepared using either Millipore Milli-Q <sup>R</sup> or equivalent deionized water and reagent grade chemicals according to EPA acute toxicity test manual) or deionized water combined with mineral water to appropriate hardness.
14. Dilution factor	$\geq 0.5$
15. Number of dilutions <sup>3</sup>	5 plus a control. An additional dilution at the permitted effluent concentration (% effluent) is required if it is not included in the dilution series.
16. Effect measured	Mortality-no movement of body or appendages on gentle prodding
17. Test acceptability	90% or greater survival of test organisms in control solution
18. Sampling requirements	For on-site tests, samples must be used within 24 hours of the time that

they are removed from the sampling device. For off-site tests, samples must first be used within 36 hours of collection.

19. Sample volume required

Minimum 1 liter

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Footnotes:

<sup>1</sup> Adapted from EPA/600/4-90/027F.

<sup>2</sup> Standard prepared dilution water must have hardness requirements to generally reflect the characteristics of the receiving water.

<sup>3</sup> When receiving water is used for dilution, an additional control made up of standard laboratory dilution water (0% effluent) is required.

**EPA NEW ENGLAND RECOMMENDED TEST CONDITIONS FOR THE  
FATHEAD MINNOW (PIMEPHALES PROMELAS) 48 HOUR ACUTE TEST<sup>1</sup>**

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1. Test Type	Static, non-renewal
2. Temperature (°C):	20 ± 1 ° C or 25 ± 1°C
3. Light quality:	Ambient laboratory illumination
4. Photoperiod:	16 hr light, 8 hr dark
5. Size of test vessels:	250 mL minimum
6. Volume of test solution:	Minimum 200 mL/replicate
7. Age of fish:	1-14 days old and age within 24 hrs of the others
8. No. of fish per chamber	10 (not to exceed loading limits)
9. No. of replicate test vessels per treatment	4
10. Total no. organisms per concentration:	40
11. Feeding regime:	Light feeding using concentrated brine shrimp nauplii while holding prior to initiating the test as per manual

12. Aeration:	None, unless dissolved oxygen (D.O.) concentration falls below 4.0 mg/L, at which time gentle single bubble aeration should be started at a rate of less than 100 bubbles/min. (Routine D.O. check is recommended.)
13. Dilution water: <sup>2</sup>	Receiving water, other surface water, synthetic soft water adjusted to the hardness and alkalinity of the receiving water (prepared using either Millipore Milli-Q <sup>R</sup> or equivalent deionized and reagent grade chemicals according to EPA acute toxicity test manual) or deionized water combined with mineral water to appropriate hardness.
14. Dilution factor	$\geq 0.5$
15. Number of dilutions <sup>3</sup>	5 plus a control. An additional dilution at the permitted effluent concentration (% effluent) is required if it is not included in the dilution series.
16. Effect measured	Mortality-no movement on gentle prodding
17. Test acceptability	90% or greater survival of test organisms in control solution
18. Sampling requirements	For on-site tests, samples must be used within 24 hours of the time that they are removed from the sampling device. For off-site tests, samples are used within 36 hours of collection.
19. Sample volume required	Minimum 2 liters

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Footnotes:

- <sup>1</sup> Adapted from EPA-600/4-90/027F.
- <sup>2</sup> Standard dilution water must have hardness requirements to generally reflect characteristics of the receiving water.
- <sup>3</sup> When receiving water is used for dilution, an additional control made up of standard laboratory dilution water (0% effluent) is required.

## VI. CHEMICAL ANALYSIS

At the beginning of a static acute toxicity test, pH, total residual chlorine, conductivity, and temperature must be measured in the highest effluent concentration and the dilution water. Dissolved oxygen, pH and temperature are also measured at 24 and 48 hour intervals. It is also recommended that total alkalinity and total hardness be measured in the control and highest effluent concentration at the beginning of the test. The following chemical analyses shall be performed for each sampling event.

<u>Parameter</u>	<u>Effluent</u>	<u>Diluent</u>	<u>Minimum Quantification Level (mg/L)</u>
Hardness <sup>*1</sup>	x	x	0.5
Alkalinity	x	x	2.0
pH	x	x	---
Specific Conductance	x	x	---
Total Solids and Suspended Solids	x	x	---
Ammonia	x	x	0.1
Total Organic Carbon	x	x	0.5
Total Residual Chlorine (TRC) <sup>*2</sup>	x	x	0.05
Dissolved Oxygen	x	x	1.0
<u>Total Metals</u>			
Cd	x	x	0.001
Cr	x	x	0.005
Pb	x	x	0.005
Cu	x	x	0.0025
Zn	x	x	0.0025
Ni	x	x	0.004
Al	x	x	0.02
Mg, Ca	x	x	0.05



Superscripts:

- \*1 Method 2340 B (hardness by calculation) from APHA (1992) Standard Methods for the Examination of Water and Wastewater. 18th Edition.
- \*2 Either of the following methods the 18th Edition of the APHA Standard Methods for the Examination of Water and Wastewater must be used for Total Residual Chlorine analyses:  
Method 4500-CL E Low Level Amperometric Titration Method (the preferred method); or  
Method 4500-CL G DPD Colorimetric Method  
or use USEPA Manual of Methods Analysis of Water and Wastes, Method 330.5

## **VII. TOXICITY TEST DATA ANALYSIS**

### LC50 Median Lethal Concentration (Determined at 48 Hours)

Methods of Estimation:

- Probit Method
- Spearman-Kärber
- Trimmed Spearman-Kärber
- Graphical

See the flow chart in Figure 6 on p. 77 of EPA 600/4-90/027F for appropriate method to use on a given data set.

### No Observed Acute Effect Level (NOAEL)

See the flow chart in Figure 13 on p. 94 of EPA 600/4-90/027F.

## **VIII. TOXICITY TEST REPORTING**

A report of the results will include the following:

- Description of sample collection procedures, site description;
- Names of individuals collecting and transporting samples, times and dates of sample collection and analysis on chain-of-custody; and
- General description of tests: age of test organisms, origin, dates and results of standard toxicant tests; light and temperature regime; other information on test conditions if different than procedures recommended. Reference toxicant test data should be included.
- All chemical/physical data generated. (Include minimum detection levels and minimum quantification levels.)

- Raw data and bench sheets.
- Provide a description of dechlorination procedures (as applicable).
- Any other observations or test conditions affecting test outcome.